



Development and validation of a rapid and sensitive UPLC–MS/MS method for determination of uracil and dihydrouracil in human plasma

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ABSTRACT

Quantification of the endogenous dihydropyrimidine dehydrogenase (DPD) substrate uracil (U) and the reaction product dihydrouracil (UH₂) in plasma might be suitable for identification of patients at risk of fluoropyrimidine-induced toxicity as a result of DPD deficiency. In this paper, we describe the development and validation of a rapid and sensitive ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) assay for quantification of U and UH₂ in human plasma.

Analytes were extracted by protein precipitation, chromatographically separated on an Acquity UPLC® HSS T3 column with gradient elution and analyzed with a tandem mass spectrometer equipped with an electrospray ionization source. U was quantified in the negative ion mode and UH₂ in the positive ion mode. Stable isotopes for U and UH₂ were used as internal standards.

Total chromatographic run time was 5 min. Validated concentration ranges for U and UH₂ were from 1 to 100 ng/mL and 10 to 1000 ng/mL, respectively. Inter-assay bias and inter-assay precision for U were within ±2.8% and ≤12.4%. For UH₂, inter-assay bias and inter-assay precision were within ±2.9% and ≤7.2%. Adequate stability of U and UH₂ in dry extract, final extract, stock solution and plasma was demonstrated. Stability of U and UH₂ in whole blood was only satisfactory when stored up to 4 hours at 2–8 °C, but not at ambient temperatures.

An accurate, precise and sensitive UPLC–MS/MS assay for quantification of U and UH₂ in plasma was developed. This assay is now applied to support clinical studies with fluoropyrimidine drugs.

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1. INTRODUCTION

Chemotherapeutic agents belonging to the group of fluoropyrimidines are the mainstay for treatment of colorectal, breast and gastric cancer. Fluoropyrimidine-based chemotherapeutic regimens usually involve administration of intravenous 5-fluorouracil (5-FU) or its orally available pre-prodrug capecitabine. After oral administration, capecitabine is rapidly and almost completely absorbed via the gastrointestinal tract and converted to 5-FU through a three-step enzymatic cascade. Approximately 80% of 5-FU is catabolized to inactive metabolites by the enzyme dihydropyrimidine dehydrogenase (DPD) [1–3]. A small fraction of 5-FU

follows a complex route of intracellular metabolism, which eventually leads to formation of active metabolites. The active metabolites are misincorporated in RNA and DNA and inhibit the enzyme thymidylate synthase, thereby inducing cell death.

Clinical application of fluoropyrimidines is seriously limited by poorly predictable treatment-related toxicity. Approximately 15–30% of patients who are treated with fluoropyrimidines will suffer from severe (≥ National Cancer Institute Common Toxicity Criteria grade 3) toxicity [4]. Treatment-related death is observed in approximately 0.5–1% of patients [5].

Several studies have been performed to examine the relationship between fluoropyrimidine-induced toxicity and DPD phenotype. The most often applied phenotyping approach is based on *ex vivo* quantification of DPD activity in peripheral blood mononuclear cells (PBMCs) [6–8]. The DPD activity in PBMCs has been associated with systemic clearance of 5-FU [9] and

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fluoropyrimidine-induced toxicity [10–12]. Although promising, determination of DPD activity in PBMCs is laborious, expensive and requires the use of radioisotopes, thereby limiting the clinical applicability of this method.

An alternative and promising DPD phenotyping approach is quantification of the endogenous DPD substrate uracil (U) and the reaction product dihydrouracil (UH₂). Several bioanalytical methods for quantification of U and UH₂ in plasma [13–25], urine [14,26–31], and saliva have been described [32]. The reported bioanalytical methods are based on high performance liquid chromatography (HPLC) coupled with ultraviolet spectrophotometry [15–17,20–24,32,33], or tandem mass spectrometry (MS/MS) [13,14,17,19,25–30], or employ gas chromatography – MS/MS [31]. Clinical relevance of U and UH₂ levels has most often been studied using the plasmatic matrix. Pre-therapeutic UH₂/U plasma ratios showed good correlation with clearance of 5-FU [34,35] and fluoropyrimidine-induced toxicity [18,34,36–38]. Therefore, upfront determination of U and UH₂ levels in plasma is an attractive and promising approach for improved identification of patients at risk of fluoropyrimidine-induced toxicity.

Previously described bioanalytical methods for U and UH₂ in plasma require extensive sample pre-treatment consisting of protein precipitation followed by liquid/liquid extraction [13,14,19–25,39], or the use of solid phase extraction [16–18], and sometimes long analytical run times (30–90 min) [16,17,20–24]. Furthermore, method validation was shown to be challenging because blank, U and UH₂ free, plasma is not available. Most described methods have therefore been validated using bovine serum albumin as a surrogate matrix for plasma or were validated using the standard addition method [13,14,16,17,19,24,25], which hampers adequate examination of matrix effects and assay sensitivity, respectively. Sistonen et al. reported that ranges of U and UH₂ plasma levels were highly variable among published studies, and suggested that this could, at least partly, be attributed to variability in method development and validation [36]. Altogether, these results indicate that development of an accurate, sensitive and robust assay for U and UH₂ in plasma is not straightforward.

Our aim was to develop and validate a rapid and sensitive bioanalytical method for quantification of U and UH₂ in human plasma. We applied ultra-performance liquid chromatography (UPLC)–MS/MS for optimal chromatographic separation, short run time and adequate sensitivity and selectivity. This method is the first to describe sample pre-treatment only by protein precipitation. Validation experiments were performed using both the original matrix and dialyzed (blank) human plasma. The applicability of the developed method is demonstrated by the quantitative analysis of U and UH₂ in plasma samples.

2. MATERIALS AND METHODS

2.1. Chemicals

U and UH₂ were purchased from Toronto Research Chemicals (North York, ON, Canada). ¹⁵N₂-labeled U and Phosphate Buffered Saline (PBS) were purchased from Sigma (St. Louis, MO, USA). ¹³C₄,¹⁵N₂-labeled UH₂ was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). UPLC-grade acetonitrile, methanol, formic acid and water were purchased from Biosolve Ltd (Valkenswaard, The Netherlands). Distilled water was purchased from B. Braun Medical (Melsungen, Germany). Dimethylsulfoxide (DMSO) was obtained from Merck (Darmstadt, Germany). Control human heparinized plasma was purchased from Bioreclamation (Hicksville, NY, USA).

2.2. Preparation of calibration standards and quality control samples

Stock solutions, which were used for preparation of calibration standards, containing 1 mg/mL of U and UH₂, were prepared in DMSO for both analytes. Separate stock solutions for quality control (QC) samples were prepared with U and UH₂ diluted in DMSO, also at concentrations of 1 mg/mL. Working solutions were obtained by diluting U and UH₂ stock solutions with water. For the internal standards, separate stock solutions containing the stable isotopes U-¹⁵N₂ and UH₂-¹³C₄-¹⁵N₂ were prepared at concentrations of 1 mg/mL in DMSO. An internal standard working solution was prepared by diluting stock solutions in 0.1% (v/v) formic acid in water up to concentrations of 1,000 and 10,000 ng/mL for U-¹⁵N₂ and UH₂-¹³C₄-¹⁵N₂ respectively. All stock and working solutions were stored at –20 °C.

Blank matrix was obtained by removing endogenous U and UH₂ from control human plasma. Therefore, control human plasma was dialyzed using Slide-A-Lyzer Dialysis Cassettes with a molecular weight cut-off of 2 kDa (Thermo Fisher Scientific, Rockford, IL, USA). Dialysis was performed by placing dialysis cassettes, which were filled with 12 mL control human plasma, in 2 L of 0.01 M PBS in distilled water under magnetic stirring at room temperature. After 0.5, 2, and 4 hours, the PBS solution was discarded and replaced by 2 L of fresh PBS. Dialysis continued overnight and the obtained blank plasma was used for preparation of calibration and QC samples. After preparation, the blank plasma was stored at –70 °C.

For preparation of calibration standards, a volume of 15 µL each working solution was added to 285 µL blank control plasma. Calibration standards containing U and UH₂ were freshly prepared at concentrations of 1, 2.5, 10, 40, 80 and 100 ng/mL for U and 10, 25, 100, 400, 800 and 1000 ng/mL for UH₂. QC samples were freshly prepared by adding 15 µL of QC working solutions to 285 µL blank control plasma. Concentrations at QC lower limit of quantification (LLOQ), low, mid and high levels were 1, 3, 25 and 75 ng/mL for U and 10, 30, 250 and 750 ng/mL for UH₂, respectively.

2.3. Sample pre-treatment

A volume of 20 µL internal standard working solutions was added to 300 µL of plasma (or control dialyzed plasma in case of calibration standards and QC samples). Plasma proteins were precipitated by adding 900 µL of methanol:acetonitrile (50:50, v/v). After a 10 s vortex spin, samples were shaken for 10 min at 1250 rpm. Next, the samples were centrifuged at 14,000 g for 10 min at room temperature. Clear supernatants were collected and evaporated under a gentle stream of nitrogen gas at 40 °C. After approximately 45 minutes, dry extracts were obtained. The dry extracts were reconstituted with 100 µL of 0.1% (v/v) formic acid in water, vortex-mixed for 10 s, and were centrifuged at 14,000 g for 10 min at 4 °C. Clear final extracts were transferred to 96-well plates (350 µL; Waters, Milford, MA, USA). A volume of 5 µL was injected into the UPLC-MS/MS system.

2.4. Liquid chromatography and mass spectrometry

U and UH₂ plasma concentrations were determined using an Acquity UPLC system (Waters, Milford, MA, USA) coupled to a QTrap 5500 triple quadrupole spectrometer (Sciex, Framingham, MA, USA). Chromatographic separation was achieved on an Acquity UPLC HSS T3 column (150 × 2.1 mm ID, particle size 1.8 µm; Waters, Milford, MA, USA). The temperature within the autosampler was set to 5 °C and the column temperature was maintained at 30 °C. Mobile phase A consisted of 0.1% (v/v) formic acid in UPLC-grade water and mobile phase B was 0.1% (v/v) formic acid in UPLC-grade acetonitrile. Gradient elution was applied at a flow

Table 1
Mass spectrometer settings for the analysis of uracil and dihydrouracil in human plasma.

Detector parameters	Setting			
Ion source	Turbo Ion Spray			
Ionization modes	Negative for U and positive for UH ₂			
Ion spray voltages	–4500/5000 V			
Entrance potential	–10/10 V			
Temperature	700 °C			
Gas 1 (nebulizer)	50 a.u.			
Gas 2	50 a.u.			
Collision gas	6 a.u.			
Curtain gas	40 a.u.			
Analyte specific parameters	U	IS U	UH ₂	IS UH ₂
Parent ion (m/z)	110.9	112.9	114.9	120.9
Product ion (m/z)	42.0	43.0	55.0	58
Declustering potential (V)	–75	–75	86	86
Collision energy (V)	–16	–16	15	15
Collision cell exit potential (V)	–19	–19	10	10
Dwell time (ms)	75	75	75	75
Typical retention time (min)	2.80	2.80	2.65	2.65

Abbreviations: a.u., arbitrary units; IS, internal standard; U, uracil; UH₂, dihydrouracil.

rate of 0.3 mL/min. The following gradient was used: 0% B from 0–3.0 min, 0–90% B from 3.0–3.2 min, 90% B from 3.2–3.7 min, 0% B from 3.7–5 min. The Qtrap 5500 mass spectrometer was equipped with a turbo spray ion source and operated in the negative ion mode for detection of U and in the positive ion mode UH₂. The analytes were detected in multiple reaction monitoring (MRM) mode. An overview of applied mass spectrometer settings, including analyte specific settings, is shown in Table 1. Analyst software (Sciex; version 1.5.2) was used to control the UPLC-MS/MS system and for data processing.

2.5. Validation procedures

2.5.1. Linearity

Calibration standards (six non-zero calibration standards, a blank standard and a standard exclusively spiked with internal standard) were prepared in duplicate in dialyzed control human plasma in three independent analytical runs. For both analytes, the linear regression of the peak area ratio (analyte/internal standard) versus the concentration was weighted $1/x^2$. Calibration concentrations were back-calculated from the calibration lines. Subsequently, deviations from the nominal concentrations were determined. Back-calculated concentrations should not deviate from nominal concentrations by more than $\pm 20\%$ at the LLoQ level and $\pm 15\%$ for higher concentrations [40,41].

2.5.2. Accuracy and precision

In three consecutive analytical runs, five replicates of QC samples were prepared in dialyzed control human plasma at QC LLoQ, low, medium and high concentrations and analyzed. Inter-assay bias and inter-assay precision were calculated in order to examine accuracy and precision of this bioanalytical assay. Inter- and intra-assay accuracies were considered acceptable in case bias was within $\pm 20\%$ at the LLoQ level and $\pm 15\%$ for other QC concentrations. Inter- and intra-assay precisions $\leq 20\%$ were considered adequate at the LLoQ level and $\leq 15\%$ for other concentrations [40,41].

2.5.3. Carry-over

Carry-over was assessed in duplicate within one analytical run by analyzing peak responses in double blank QC samples after injection of the highest calibration sample. The response in double blank samples should not be greater than 20% of the peak response of U

and UH₂ at the LLoQ level and 5% of the response of the internal standards [40,41].

2.5.4. Sample preparation for assessing matrix factor, recovery, and selectivity

The analytes U and UH₂ are naturally present in human plasma at concentrations of approximately 10 ng/mL and 100 ng/mL, respectively [36]. However, the stable isotopes U-¹⁵N₂ and UH₂-¹³C₄-¹⁵N₂ are not occurring in human plasma and have similar chemical properties to U and UH₂. Therefore, control human plasma was spiked with U-¹⁵N₂ and UH₂-¹³C₄-¹⁵N₂ and quantified using U and UH₂ as internal standards for examination of matrix factor, recovery and selectivity. For these purposes, working solutions containing the stable isotopes U-¹⁵N₂ and UH₂-¹³C₄-¹⁵N₂ were prepared in water. Samples were prepared by adding 15 μ L of these working solutions to 285 μ L non-dialyzed heparinized control human plasma (QC-IS samples). Concentrations at the QC-IS low and high level were 3 and 75 ng/mL for U-¹⁵N₂ and 30 and 750 ng/mL for UH₂-¹³C₄-¹⁵N₂. Calibration standards containing U-¹⁵N₂ and UH₂-¹³C₄-¹⁵N₂ were freshly prepared at concentrations of 1, 2.5, 10, 40, 80 and 100 ng/mL for U-¹⁵N₂ and 10, 25, 100, 400, 800 and 1,000 ng/mL for UH₂-¹³C₄-¹⁵N₂. An internal standard working solution was prepared with U and UH₂ at concentrations of 1,000 and 10,000 ng/mL, respectively. Addition of this internal standard working solution to QC-IS samples resulted in U and UH₂ levels that greatly exceeded endogenous U and UH₂ levels, thereby avoiding the introduction of a bias in the quantification of U-¹⁵N₂ and UH₂-¹³C₄-¹⁵N₂ in these samples. Sample pre-treatment was according to the procedure described above.

2.5.5. Matrix factor and recovery

Matrix effects were investigated at QC-IS low and high levels in triplicate using the same batch of human control plasma. The absolute matrix factor was determined by calculating the ratio of the peak area in the presence of matrix ions to the peak area in absence of matrix. Samples without matrix ions consisted of neat solutions in 0.1% formic acid in water with analyte concentrations at QC-IS low and high level. For samples including matrix ions, dry extracts of blank control plasma were spiked with the same neat solutions. The relative matrix factor was determined by dividing the matrix factor of the analytes through the matrix factors of the corresponding internal standards.

Overall and protein precipitation (PP) recovery were determined at QC-IS low and high levels in triplicate. The PP recovery was

calculated by comparing peak areas of processed samples with peak areas of blank control samples that were reconstituted with neat solutions of analytes (representing 100% recovery). Overall recovery was determined by comparing analyte peak areas of processed samples with peak areas of analyte in absence of matrix ions.

The relative standard deviation (RSD) values of relative matrix factor and PP recovery should be $\leq 15\%$ [40,41].

2.5.6. Selectivity

Six different batches of human heparinized control plasma were spiked at QC-IS low level. Selectivity was evaluated by determining endogenous interferences for U- $^{15}\text{N}_2$ and UH_2 - $^{13}\text{C}_4$ - $^{15}\text{N}_2$ in double blank samples in relation to peak responses of QC-IS low samples. Endogenous interference should be equal to or less than 15% of the peak area of QC-IS low samples.

2.5.7. Stability

The stability of U and UH_2 stock solutions was examined after storage for 148 days at -20°C . Short-term stability in plasma was determined in triplicate by analyzing QC-IS low and high samples after a storage period of 4 hours at ambient temperatures. Long-term stability in plasma was assessed by re-analysis of study samples. These study samples were collected from patients who participated in a phase I clinical study that was approved by the Ethics Committee of the Antoni van Leeuwenhoek Hospital, Amsterdam, The Netherlands. After initial analysis, five samples with concentrations close to QC low and medium levels were selected for re-analysis after storage for 93 days at -70°C . Stability of U and UH_2 in whole blood was examined for 0.5, 1, 2, 4 and 6 hours at ambient temperatures or 2 – 8°C . For this purpose, peripheral blood was collected from three volunteers in heparinized tubes. Stability in dry extract and final extract were determined after storage of five QC samples at low and high level for 5 days at 2 – 8°C . Stability of analytes was considered acceptable if 85–115% of the initial concentration was recovered.

3. RESULTS AND DISCUSSION

3.1. Assay development

Sample pre-treatment and analytical run time had to be adequate and efficient for rapid and high-throughput quantification of U and UH_2 in human plasma. The most frequently described sample pre-treatment procedure for determination of U and UH_2 consists of ammonium sulfate precipitation followed by liquid-liquid extraction with a mixture of ethyl acetate and isopropanol [14]. This two-step pre-treatment has been shown to be adequate, but limits high sample throughput because it is rather laborious. In order to accelerate and simplify sample pre-treatment, a procedure consisting of protein precipitation with a mixture of methanol and acetonitrile (50:50, v/v) was successfully applied. The methanol:acetonitrile mixture was added to plasma with a ratio of 3:1. After protein precipitation, clear supernatants were quickly evaporated under a stream of nitrogen, reconstituted in mobile phase A, and transferred to a 96-well plate. The use of 96-well plates allowed for analysis of large batches of samples.

The analytes U and UH_2 (Fig. S1) are highly polar. In order to obtain adequate analyte retention, an analytical column that remains stable at highly aqueous mobile phase conditions is warranted. Others have shown that chromatographic separation of U and UH_2 was challenging. Sparidans et al. and Buchel et al. used a reversed-phase Atlantis dC18 column (Waters) and obtained adequate peak separation only when the column was cooled with ice or maintained at 5°C , respectively [13,26]. We applied reversed-phase chromatography and used the Acquity UPLC[®] HSS T3 column. Using this analytical column, and gradient elution with 0.1% formic acid in

water and 0.1% formic acid acetonitrile, adequate analyte retention and separation were obtained, without the need of refrigeration of the column. Total analytical run time was only 5 minutes and typical retention times of U and UH_2 were 2.80 and 2.65 minutes. Flow injection analysis was applied for optimization of the source settings and the analyte-specific conditions. In order to obtain largest signal-to-noise ratios, the mass spectrometer was utilized in the negative ion mode for quantification of U and in the positive ion mode for quantification of UH_2 . Optimized detector and analyte specific settings are presented in Table 1. Representative chromatograms of a blank QC sample, QC LLoQ sample and study sample are shown in Fig. 1. Chromatographic analysis of the study sample demonstrated peaks at 2.2 and 2.9 min in the chromatograms for U and UH_2 , respectively (Fig. 1). These peaks were not present in the chromatograms of the QC samples. The compounds that elute at 2.2 and 2.9 min were possibly removed from control plasma during the dialysis procedure, which could explain why the peaks do not appear in the chromatograms of the QC samples.

3.2. Assay validation

3.2.1. Linearity

The assay was linear over a concentration range of 1–100 ng/mL for U and 10–1000 ng/mL for UH_2 . Correlation coefficients of the calibration curves were 0.998 or higher for U and 0.995 or higher for UH_2 . The average slope of the U calibration curve was 0.0131 with a RSD of 9.4%. For UH_2 , the average slope of the calibration curve was 0.00177 with a RSD of 3.5%. Intersects of the U and UH_2 calibration curves were within ± 0.00312 and ± 0.00181 , respectively. Deviations of back-calculated concentrations from nominal concentrations were between -2.0 and 1.6% for U with RSD values $\leq 7.1\%$. Regarding UH_2 , deviations of back-calculated concentrations were between -2.0 and 3.2% with RSD values $\leq 10.3\%$. Relative differences between the back-calculated and nominal concentrations are shown in Fig. S2. The acceptance criteria concerning linearity were met for both analytes.

3.2.2. Accuracy and precision

The results regarding inter-assay bias and precision are summarized in Table 2. Intra-assay biases were within -1.3 and 5.7% at the QC LLoQ level and within $\pm 7.3\%$ at the higher QC levels. Intra-assay precisions were within 5.3 and 19.7% at the QC LLoQ level and $\leq 7.4\%$ at the higher QC levels. One measure for U at QC LLoQ was considered an outlier based on the Dixon's Q test for outliers and excluded from the analysis. The results met the predefined criteria for adequate accuracy and precision.

3.2.3. Carry-over

Carry-over for U was $\leq 9.8\%$ of the signal at the corresponding LLoQ and for no carry-over was observed for UH_2 . For U- $^{15}\text{N}_2$ and UH_2 - $^{13}\text{C}_4$ - $^{15}\text{N}_2$, carry-over was $\leq 0.2\%$ and $\leq 0.1\%$, respectively. It was concluded that the carry-over test fulfilled the predefined criteria.

3.2.4. Matrix factor and recovery

Table 3 shows the results of matrix factor and recovery analyses. The results indicate that U- $^{15}\text{N}_2$ and UH_2 - $^{13}\text{C}_4$ - $^{15}\text{N}_2$ are subject to ion suppression, and that the role of matrix effects is effectively minimized by the internal standards U and UH_2 . Moreover, these results show that ion suppression for U and UH_2 was observed in the same extent as for the stable isotopes. Therefore, the effect of ion suppression on quantification of U and UH_2 in human plasma samples can be minimized by the use of internal standards U- $^{15}\text{N}_2$ and UH_2 - $^{13}\text{C}_4$ - $^{15}\text{N}_2$.

Mean PP recovery for U- $^{15}\text{N}_2$ and UH_2 - $^{13}\text{C}_4$ - $^{15}\text{N}_2$ ranged between 97.0% and 114% (Table 3). Relatively low overall recovery

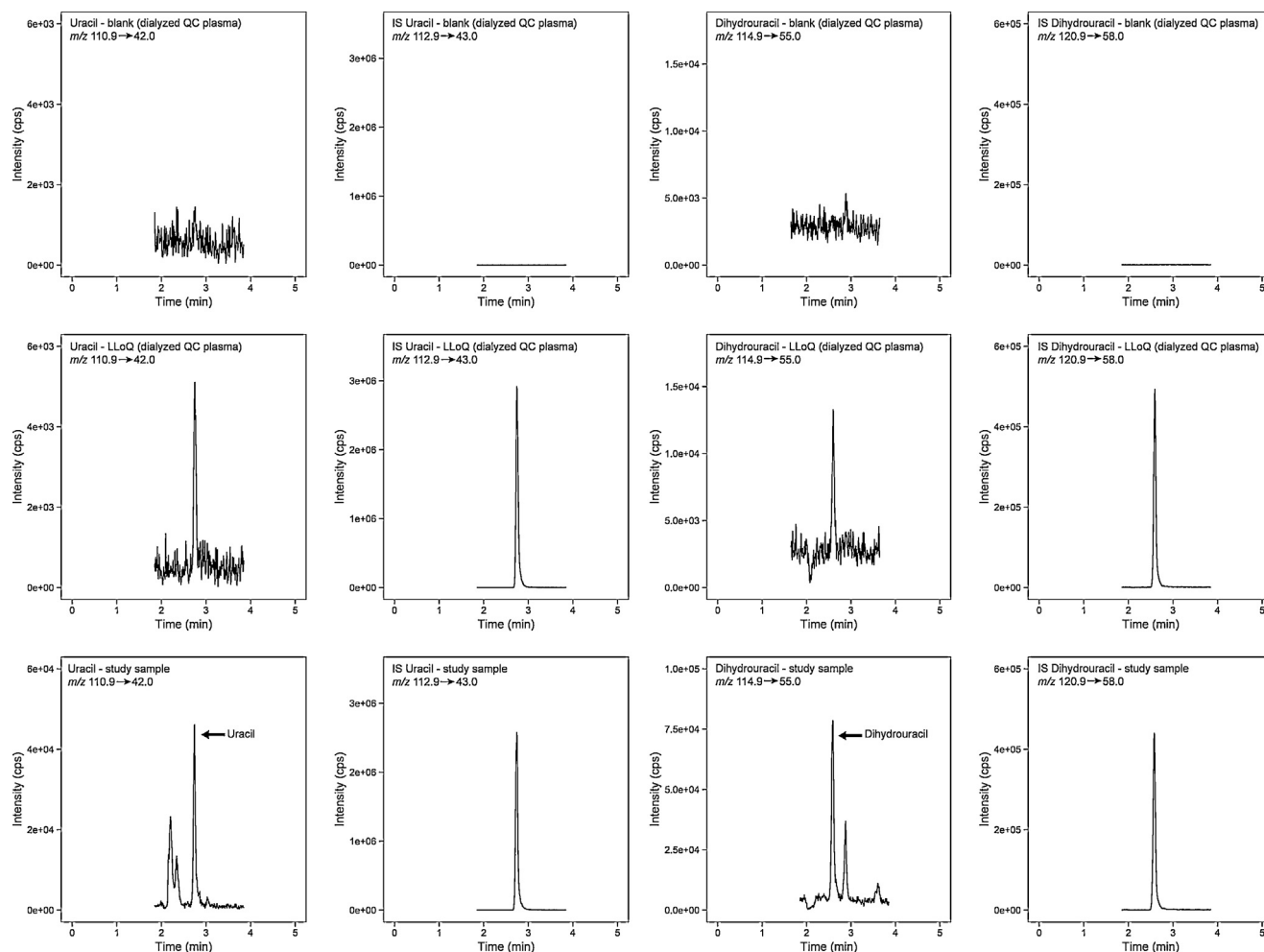


Fig. 1. Representative chromatograms of uracil (U) and dihydrouracil (UH₂) and internal standards (IS) for blank (dialyzed) samples, quality control (QC) LLoQ sample and study sample. The U and UH₂ plasma concentrations in the study sample were 11.7 ng/mL and 113 ng/mL, respectively.

Table 2

Assay performance data for the analysis of uracil and dihydrouracil in human plasma at validated quality control concentration levels.

Analyte	QC	Nominal concentration (ng/mL)	Average measured concentration (ng/mL)	Inter-assay bias (%)	Inter-assay precision (%)	Number of replicates
U	LLoQ	1	1.02	1.9	12.4	14 ^a
	Low	3	3.07	2.2	5.4	15
	Medium	25	24.8	−0.9	5.6	15
	High	75	77.1	2.8	5.1	15
UH ₂	LLoQ	10	10.3	2.9	7.2	15
	Low	30	29.6	−1.3	6.4	15
	Medium	250	257	2.8	4.3	15
	High	750	771	2.8	5.4	15

Abbreviations: QC, quality control; U, uracil; UH₂, dihydrouracil; LLoQ, lower limit of quantification.

^a One value was considered a statistical outlier using the Dixon's Q test and excluded from the analysis.

Table 3

Matrix factor and recovery for stable isotopes of uracil and dihydrouracil.

Analyte ^a	Nominal concentration	Absolute matrix factor	RSD (%)	Relative matrix factor	RSD (%)	Protein precipitation recovery (%)	RSD (%)	Overall recovery (%)	RSD (%)
U- ¹⁵ N ₂	3	0.476	6.7	0.856	6.5	97.9	5.3	46.2	5.7
	75	0.456	1.0	0.906	7.0	100	0.8	45.4	0.4
UH ₂ - ¹³ C ₄ - ¹⁵ N ₂	30	0.724	6.0	1.00	7.9	97.9	5.6	70.7	1.7
	750	0.686	2.2	0.893	3.0	114	1.7	78.1	2.2

Abbreviations: RSD, relative standard deviation; U, uracil; UH₂, dihydrouracil.

^a Uracil and dihydrouracil are naturally present in control human plasma. Therefore, control human plasma was spiked with the stable isotopes U-¹⁵N₂ and UH₂-¹³C₄-¹⁵N₂ and quantified using U and UH₂ as internal standards.

Table 4
Stability data for uracil and dihydrouracil.

Analyte	Condition	Matrix	Initial concentration (ng/mL)	Observed concentration(ng/mL)	Deviation(%)	RSD(%)
U	–20 °C, 148 days	Stock solution	1.00×10^6	0.96×10^6	–3.9	7.1
		Plasma	3.00	3.00	0.0	5.5
			75.0	72.2	–3.8	3.7
	–70 °C, 93 days ^a	Plasma	11.8	11.8	–0.5	N/A
			29.6	27.5	–7.3	N/A
	2–8 °C, 5 days	Dry extract	3.00	3.01	0.4	6.2
			75.0	76.9	2.5	4.9
	2–8 °C, 5 days	Final extract	3.00	3.02	0.6	8.2
			75.0	75.3	0.4	4.3
UH ₂	–20 °C, 148 days	Stock solution	1.00×10^6	1.05×10^6	4.9	2.1
		Plasma	30.0	28.7	–4.4	6.8
			750	759	1.2	3.9
	–70 °C, 93 days ^a	Plasma	82.0	73.1	–10.1	N/A
			282	257	–8.8	N/A
	2–8 °C, 5 days	Dry extract	30.0	32.1	6.9	3.3
			750	751	0.2	6.0
	2–8 °C, 5 days	Final extract	30.0	31.3	4.5	4.3
			750	733	–2.3	2.8

Abbreviations: RSD, relative standard deviation; N/A, not applicable; U, uracil; UH₂, dihydrouracil.

^a Five study samples with initial U and UH₂ levels close to QC Low and QC Medium levels were selected and re-analyzed after storage at –70 °C for 93 days. The deviation from the initial measured concentrations is shown.

of both U-¹⁵N₂ and UH₂-¹³C₄-¹⁵N₂ is the result of ion suppression (Table 3), since there was a pronounced matrix effect identified.

The RSD values of relative matrix factor and PP recovery were ≤7.9% and ≤5.6%, respectively, and fulfilled predefined criteria.

3.2.5. Selectivity

Endogenous interferences did not exceed 0.3% of the peak area of U-¹⁵N₂ at the QC-IS low level. There was no endogenous interference detected for UH₂-¹³C₄-¹⁵N₂. Based on these data, selectivity for the stable isotopes was considered acceptable.

Selectivity for U and UH₂ could not be determined because these analytes are endogenous compounds and present in the matrix. Dialyzed control human plasma is free of U and UH₂. However, during the preparation of dialyzed control plasma, molecules with a molecular weight below 2 kDa are eliminated from the matrix. Consequently, molecules that potentially interfere with quantification of U and UH₂ could be diminished by dialysis of the control human plasma. The use of dialyzed control human plasma was therefore not considered a suitable matrix for determination of selectivity of this assay for U and UH₂.

3.2.6. Stability

Results of stability experiments are shown in Table 4. Both U and UH₂ were stable at –20 °C for at least 148 days. These analytes also showed adequate stability in dry extracts and final extracts that were stored at 2–8 °C for 5 days. Short-term stability of U and UH₂ in plasma was examined by spiking the stable isotopes U-¹⁵N₂ and UH₂-¹³C₄-¹⁵N₂ at QC-IS low and high levels. After 4 hours at ambient temperatures, there was no significant change in U-¹⁵N₂ and UH₂-¹³C₄-¹⁵N₂ levels detected. Therefore, it can be concluded that also U and UH₂ have acceptable stability in plasma for at least 4 hours at ambient temperatures. Long-term stability of U and UH₂ in plasma was examined by re-analysis of study samples. Both analytes showed adequate stability in plasma that was stored at –70 °C for 93 days. All five samples that were re-analyzed for stability assessment at the QC low level for U and QC medium level of UH₂ showed a relative change to initial measurement that was within ±15%. Four out of five samples that were selected for long-term stability assessment for U at QC medium level and UH₂ at the QC low level showed a deviation that was within ±15%.

Stability of U and UH₂ in whole blood was determined both at ambient temperatures and at 2–8 °C. The concentration of U in plasma showed a large and variable percent increase from

baseline after storage of whole blood at ambient temperatures (Fig. S3). Increase in U plasma levels was $13.8 \pm 5.0\%$ after storage of whole blood for 4 hours at 2–8 °C and was $21.2 \pm 2.1\%$ after storage for 6 hours. The relative increase in UH₂ plasma levels was less affected by storage of whole blood at both temperature conditions (Fig. S3). After storage of whole blood for 6 hours, relative change in UH₂ plasma levels were $-6.9 \pm 4.9\%$ at 2–8 °C and $9.8 \pm 12.2\%$ at ambient temperatures. Thus, stability of UH₂ seems most adequately assured when whole blood was stored at 2–8 °C. It can be concluded that whole blood should not be stored longer than 4 hours at 2–8 °C for acceptable analysis of both U and UH₂ plasma levels. Storage of whole blood at ambient temperatures is discouraged.

These findings are in line with findings of Coudoré et al., who reported increased U and UH₂ plasma levels after storage of whole blood at ambient temperatures [19]. Increased levels of U and UH₂ that were found after storage of whole blood at ambient temperatures could be the result of *ex vivo* metabolism. The precursor of U, uridine, is present in human plasma [42], and is converted to U by uridine phosphorylase, an enzyme that is known to be expressed by many cell types, among which PBMCs [43]. In turn, formed U could be converted to UH₂ by DPD that is expressed by leukocytes and platelets [44]. Thus, *ex vivo* metabolism could lead to increased levels of UH₂ after storage of whole blood at ambient temperatures. Alternatively, U and UH₂ might be released by blood cells or transporter proteins during storage of whole blood at ambient temperatures, which could lead to increased UH₂ plasma levels.

4. CLINICAL APPLICABILITY

The described assay is currently applied for the analysis of U and UH₂ plasma levels to support a pharmacological phase I clinical study of chronomodulated capecitabine therapy in patients (<http://www.trialregister.nl>, study identifier: NTR4639). Fig. 2 shows the measured U and UH₂ plasma concentrations in a patient with colorectal cancer that participated in this study after oral administration of 900 mg capecitabine. As shown in this figure, the U and UH₂ plasma levels were within the validated ranges. Both U and UH₂ levels decreased within the first hour after administration of capecitabine, followed by an increase and subsequent stabilization. These changes in U and UH₂ plasma levels could be caused by competition between U and UH₂ and capecitabine metabolites for the same enzymes. However, more research is obviously needed to

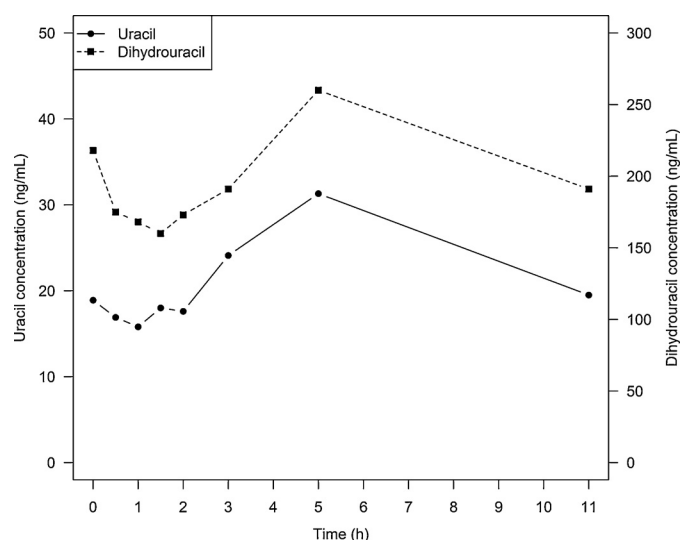


Fig. 2. Plasma concentrations of uracil and dihydrouracil in a patient with colorectal cancer following administration of 900 mg of capecitabine at the 7th day of treatment.

draw solid conclusions on this. The described assay is also applied in a large retrospective study and a prospective study (<http://www.clinicaltrials.gov>, study identifier: NCT02324452) to assess the clinical implications of U and UH_2 plasma levels for identification of patients who are DPD deficient and at risk of developing severe fluoropyrimidine-induced toxicity.

5. CONCLUSION

An accurate, precise, robust and sensitive UPLC-MS/MS assay for quantification of U and UH_2 in plasma was developed and validated. Sample pre-treatment consists only of protein precipitation, which together with short chromatographic run time, enables fast sample analysis. The validated concentration ranges are 1–100 ng/mL for U and 10–1000 ng/mL for UH_2 . Stable isotopes were used as internal standards for both analytes, enabling examination of matrix factor, recovery and selectivity without using an artificial plasma matrix. Moreover, these stable isotopes are essential for correction of matrix effects. Selectivity was not evaluated for U and UH_2 . Stability of U and UH_2 in whole blood was adequate at 2–8 °C, but not at ambient temperatures. Examined assay validation parameters fulfilled the acceptance criteria of the US Food and Drug Administration and the European Medicines Agency guidelines for method validation [40,41]. The developed method allows fast and reliable quantitative analysis of U and UH_2 in plasma samples.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jpba.2016.04.039>.

References

- [1] G.D. Heggie, J.P. Sommadossi, D.S. Cross, W.J. Huster, R.B. Diasio, Clinical pharmacokinetics of 5-fluorouracil and its metabolites in plasma, urine, and bile, *Cancer Res.* 47 (1987) 2203–2206.
- [2] C.F. Thorn, S. Marsh, M.W. Carrillo, H.L. McLeod, T.E. Klein, R.B. Altman, PharmGKB summary: fluoropyrimidine pathways, *Pharmacogenet. Genomics* 21 (2011) 237–242, <http://dx.doi.org/10.1097/FPC.0b013e328348c6f2>.
- [3] I.R. Judson, P.J. Beale, J.M. Trigo, W. Aherne, T. Crompton, D. Jones, E. Bush, B. Reigner, A human capecitabine excretion balance and pharmacokinetic study after administration of a single oral dose of ^{14}C -labelled drug, *Invest. New Drugs* 17 (1999) 49–56.
- [4] C. Twelves, A. Wong, M.P. Nowacki, M. Abt, H. Burris, A. Carrato, J. Cassidy, A. Cervantes, J. Fagerberg, V. Georgoulas, F. Hussein, D. Jodrell, P. Koralewski, H. Kröning, J. Maroun, N. Marschner, J. McKendrick, M. Pawlicki, R. Rosso, J. Schüller, J.-F. Seitz, B. Stabuc, J. Tujakowski, G. van Hazel, J. Zaluski, W. Scheithauer, Capecitabine as adjuvant treatment for stage III colon cancer, *N. Engl. J. Med.* 352 (2005) 2696–2704, <http://dx.doi.org/10.1056/NEJMoa043116>.
- [5] S.E. Mikhail, J.F. Sun, J.L. Marshall, Safety of capecitabine: a review, *Expert Opin. Drug Saf.* 9 (2010) 831–841, <http://dx.doi.org/10.1517/14740338.2010.511610>.
- [6] D. Pluim, B.A.W. Jacobs, M.J. Deenen, A.E.M. Ruijter, R.M.J.M. van Geel, A.M. Burylo, D. Meulendijks, J.H. Beijnen, J.H.M. Schellens, Improved pharmacodynamic assay for dihydropyrimidine dehydrogenase activity in peripheral blood mononuclear cells, *Bioanalysis* 7 (2015) 519–529, <http://dx.doi.org/10.4155/bio.14.304>.
- [7] D. Pluim, B.A.W. Jacobs, M.D. Krähenbühl, A.E.M. Ruijter, J.H. Beijnen, J.H.M. Schellens, Correction of peripheral blood mononuclear cell cytosolic protein for hemoglobin contamination, *Anal. Bioanal. Chem.* 405 (2013) 2391–2395.
- [8] A.B.P. van Kuilenburg, H. Van Lenthe, A. Tromp, P.C. Veltman, A.H. Van Gennip, Pitfalls in the diagnosis of patients with a partial dihydropyrimidine dehydrogenase deficiency, *Clin. Chem.* 46 (2000) 9–17.
- [9] R.A. Fleming, G. Milano, A. Thyss, M.C. Etienne, N. Renée, M. Schneider, F. Demard, Correlation between dihydropyrimidine dehydrogenase activity in peripheral mononuclear cells and systemic clearance of fluorouracil in cancer patients, *Cancer Res.* 52 (1992) 2899–2902.
- [10] A.B.P. van Kuilenburg, R. Meisma, L. Zoetekouw, A.H. Van Gennip, Increased risk of grade IV neutropenia after administration of 5-fluorouracil due to a dihydropyrimidine dehydrogenase deficiency: High prevalence of the $\text{IVS14} + 1\text{G} > \text{A}$ mutation, *Int. J. Cancer* 101 (2002) 253–258.
- [11] G. Milano, M.C. Etienne, V. Pierrefite, M. Barberi-Heyob, R. Deporte-Fety, N. Renée, Dihydropyrimidine dehydrogenase deficiency and fluorouracil-related toxicity, *Br. J. Cancer* 79 (1999) 627–630, <http://dx.doi.org/10.1038/sj.bjc.6690098>.
- [12] A.B.P. Van Kuilenburg, J. Haasjes, D.J. Richel, L. Zoetekouw, H. Van Lenthe, R.A. de Abreu, J.G. Maring, P. Vreken, A.H. van Gennip, Clinical implications of dihydropyrimidine dehydrogenase (DPD) deficiency in patients with severe 5-fluorouracil-associated toxicity: Identification of new mutations in the DPD gene, *Clin. Cancer Res.* 6 (2000) 4705–4712.
- [13] B. Büchel, P. Rhyn, S. Schürch, C. Bühr, U. Amstutz, C.R. Largiadèr, LC-MS/MS method for simultaneous analysis of uracil, 5,6-dihydrouracil, 5-fluorouracil and 5-fluoro-5,6-dihydrouracil in human plasma for therapeutic drug monitoring and toxicity prediction in cancer patients, *Biomed. Chromatogr.* 27 (2013) 7–16.
- [14] H. Jiang, J. Jiang, P. Hu, Y. Hu, Measurement of endogenous uracil and dihydrouracil in plasma and urine of normal subjects by liquid chromatography-tandem mass spectrometry, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 769 (2002) 169–176.
- [15] M.C. van Staveren, B. Theeuwes-Onok, H.-J. Guchelaar, A.B.P. van Kuilenburg, J.G. Maring, Pharmacokinetics of orally administered uracil in healthy volunteers and in DPD-deficient patients, a possible tool for screening of DPD deficiency, *Cancer Chemother. Pharmacol.* 68 (2011) 1611–1617, <http://dx.doi.org/10.1007/s00280-011-1661-5>.
- [16] R. Déporte, M. Amiard, A. Moreau, C. Charbonnel, L. Campion, High-performance liquid chromatographic assay with UV detection for measurement of dihydrouracil/uracil ratio in plasma, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 834 (2006) 170–177, <http://dx.doi.org/10.1016/j.jchromb.2006.02.046>.
- [17] R. Svobait, I. Solassol, F. Pinguet, L. Ivanauskas, J. Brès, F.M.M. Bressolle, HPLC with UV or mass spectrometric detection for quantifying endogenous uracil and dihydrouracil in human plasma, *Clin. Chem.* 54 (2008) 1463–1472, <http://dx.doi.org/10.1373/clinchem.2007.102251>.
- [18] M.H. Kristensen, P. Pedersen, J. Mejer, The value of dihydrouracil/uracil plasma ratios in predicting 5-fluorouracil-related toxicity in colorectal cancer patients, *J. Int. Med. Res.* 38 (2010) 1313–1323.
- [19] F. Coudoré, D. Roche, S. Lefevre, D. Faussot, E.M. Billaud, M.-A. Liorot, P. Beaune, Validation of an ultra-high performance liquid chromatography tandem mass spectrometric method for quantifying uracil and 5,6-dihydrouracil in human plasma, *J. Chromatogr. Sci.* 50 (2012) 877–884, <http://dx.doi.org/10.1093/chromsci/bms085>.
- [20] M.B. Garg, J.C. Sevester, J.A. Sakoff, S.P. Ackland, Simple liquid chromatographic method for the determination of uracil and dihydrouracil plasma levels: a potential pretreatment predictor of 5-fluorouracil toxicity, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 774 (2002) 223–230.

- [21] G. Remaud, M. Boisdron-Celle, C. Hameline, A. Morel, E. Gamelin, An accurate dihydrouracil/uracil determination using improved high performance liquid chromatography method for preventing fluoropyrimidines-related toxicity in clinical practice, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 823 (2005) 98–107, <http://dx.doi.org/10.1016/j.jchromb.2005.05.044>.
- [22] J. Ciccolini, C. Mercier, M.-F. Blachon, R. Favre, A. Durand, B. Lacarelle, A simple and rapid high-performance liquid chromatographic (HPLC) method for 5-fluorouracil (5-FU) assay in plasma and possible detection of patients with impaired dihydropyrimidine dehydrogenase (DPD) activity, *J. Clin. Pharm. Ther.* 29 (2004) 307–315, <http://dx.doi.org/10.1111/j.1365-2710.2004.00569.x>.
- [23] E. Gamelin, M. Boisdron-Celle, F. Larra, J. Robert, A simple chromatographic method for the analysis of pyrimidines and their dihydrogenated metabolites, *J. Liq. Chromatogr. Relat. Technol.* 20 (1997) 3155–3172, <http://dx.doi.org/10.1080/10826079708000481>.
- [24] R.Z. Hahn, A.F.A. Galarza, A. Schneider, M.V. Antunes, G. Schwartzmann, R. Linden, Improved determination of uracil and dihydrouracil in plasma after a loading oral dose of uracil using high-performance liquid chromatography with photodiode array detection and porous graphitic carbon stationary phase, *Clin. Biochem.* 48 (2015) 915–918.
- [25] I.C. César, G.F. Cunha-Júnior, R.M. Duarte Byrro, L.G. Vaz Coelho, G.A. Pianetti, A rapid HPLC-ESI-MS/MS method for determination of dihydrouracil/uracil ratio in plasma: evaluation of toxicity to 5-fluorouracil in patients with gastrointestinal cancer, *Ther. Drug Monit.* 34 (2012) 59–66.
- [26] R.W. Sparidans, T.M. Bosch, M. Jörgen, J.H.M. Schellens, J.H. Beijnen, Liquid chromatography-tandem mass spectrometric assay for the analysis of uracil, 5,6-dihydrouracil and beta-ureidopropionic acid in urine for the measurement of the activities of the pyrimidine catabolic enzymes, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 839 (2006) 45–53, <http://dx.doi.org/10.1016/j.jchromb.2006.02.016>.
- [27] H. van Lenthe, A.B.P. van Kuilenburg, T. Ito, A.H. Bootsma, A. van Cruchten, Y. Wada, A.H. van Gennip, Defects in pyrimidine degradation identified by HPLC-electrospray tandem mass spectrometry of urine specimens or urine-soaked filter paper strips, *Clin. Chem.* 46 (2000) 1916–1922.
- [28] S. Hartmann, J.G. Okun, C. Schmidt, C.-D. Langhans, S.F. Garbade, P. Burgard, D. Haas, J. Oliver Sass, W.L. Nyhan, G.F. Hoffmann, Comprehensive detection of disorders of purine and pyrimidine metabolism by HPLC with electrospray ionization tandem mass spectrometry, *Clin. Chem.* 52 (2006) 1127–1137, <http://dx.doi.org/10.1373/clinchem.2005.058842>.
- [29] Q. Sun, Urine Pyrimidine Metabolite Determination by HPLC Tandem Mass Spectrometry, *Methods Mol. Biol.* 1378 (2016) 237–242, http://dx.doi.org/10.1007/978-1-4939-3182-8_25.
- [30] C. Schmidt, U. Hofmann, D. Kohlmüller, T. Mürdter, U.M. Zanger, M. Schwab, G.F. Hoffmann, Comprehensive analysis of pyrimidine metabolism in 450 children with unspecific neurological symptoms using high-pressure liquid chromatography-electrospray ionization tandem mass spectrometry, *J. Inher. Metab. Dis.* 28 (2005) 1109–1122, <http://dx.doi.org/10.1007/s10545-005-0133-7>.
- [31] U. Hofmann, M. Schwab, S. Seefried, C. Marx, U.M. Zanger, M. Eichelbaum, T.E. Mürdter, Sensitive method for the quantification of urinary pyrimidine metabolites in healthy adults by gas chromatography-tandem mass spectrometry, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 791 (2003) 371–380.
- [32] G. Carlsson, E. Odin, B. Gustavsson, Y. Wettergren, Pretherapeutic uracil and dihydrouracil levels in saliva of colorectal cancer patients are associated with toxicity during adjuvant 5-fluorouracil-based chemotherapy, *Cancer Chemother. Pharmacol.* 74 (2014) 757–763.
- [33] M.H. Kristensen, M. Weidinger, M. Bzorek, P.L. Pedersen, J. Mejer, Correlation between thymidylate synthase gene variants, RNA and protein levels in primary colorectal adenocarcinomas, *J. Int. Med. Res.* 38 (2010) 484–497.
- [34] M. Gamelin, M. Boisdron-Celle, V. Guérin-Meyer, R. Delva, A. Lortholary, F. Genevieve, F. Larra, N. Ifrah, J. Robert, Correlation between uracil and dihydrouracil plasma ratio, fluorouracil (5-FU) pharmacokinetic parameters, and tolerance in patients with advanced colorectal cancer: A potential interest for predicting 5-FU toxicity and determining optimal 5-FU dosage, *J. Clin. Oncol.* 17 (1999) 1105–1110.
- [35] H. Jiang, J. Lu, J. Jiang, P. Hu, Important role of the dihydrouracil/uracil ratio in marked interpatient variations of fluoropyrimidine pharmacokinetics and pharmacodynamics, *J. Clin. Pharmacol.* 44 (2004) 1260–1272, <http://dx.doi.org/10.1177/0091270004268911>.
- [36] J. Sistonen, B. Büchel, T.K. Froehlich, D. Kummer, S. Fontana, M. Joerger, A.B.P. van Kuilenburg, C.R. Largiadèr, Predicting 5-fluorouracil toxicity: DPD genotype and 5,6-dihydrouracil/uracil ratio, *Pharmacogenomics* 15 (2014) 1653–1666, <http://dx.doi.org/10.2217/pgs.14.126>.
- [37] Z.W. Zhou, G.Q. Wang, D. Sen Wan, Z.H. Lu, Y.B. Chen, S. Li, G. Chen, Z.Z. Pan, The dihydrouracil/uracil ratios in plasma and toxicities of 5-fluorouracil-based adjuvant chemotherapy in colorectal cancer patients, *Chemotherapy* 53 (2007) 127–131, <http://dx.doi.org/10.1159/000099984>.
- [38] M. Boisdron-Celle, G. Remaud, S. Traore, A.L. Poirier, L. Gamelin, A. Morel, E. Gamelin, 5-Fluorouracil-related severe toxicity: a comparison of different methods for the pretherapeutic detection of dihydropyrimidine dehydrogenase deficiency, *Cancer Lett.* 249 (2007) 271–282, <http://dx.doi.org/10.1016/j.canlet.2006.09.006>.
- [39] J.G. Maring, L. Schouten, B. Greijdanus, E.G.E. de Vries, D.R.A. Uges, A simple and sensitive fully validated HPLC-UV method for the determination of 5-fluorouracil and its metabolite 5,6-dihydrofluorouracil in plasma, *Ther. Drug Monit.* 27 (2005) 25–30.
- [40] European Medicines Agency, Committee for Medicinal Products for Human Use, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf, 2011 (accessed 22.11.2015).
- [41] US Food and Drug Administration, Department of Health and Human Services, <http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf>, 2001 (accessed 22.11.2015).
- [42] T.W. Traut, Physiological concentrations of purines and pyrimidines, *Mol. Cell. Biochem.* 140 (1994) 1–22, <http://dx.doi.org/10.1007/BF00928361>.
- [43] O.H. Temmink, M. de Bruin, A.C. Laan, A.W. Turksma, S. Cricca, A.J. Masterson, P. Noordhuis, G.J. Peters, The role of thymidine phosphorylase and uridine phosphorylase in (fluoro)pyrimidine metabolism in peripheral blood mononuclear cells, *Int. J. Biochem. Cell Biol.* 38 (2006) 1759–1765, <http://dx.doi.org/10.1016/j.biocel.2006.04.007>.
- [44] A.B.P. Van Kuilenburg, H. van Lenthe, M.J. Blom, E.P. Mul, A.H. Van Gennip, Profound variation in dihydropyrimidine dehydrogenase activity in human blood cells: major implications for the detection of partly deficient patients, *Br. J. Cancer.* 79 (1999) 620–626, <http://dx.doi.org/10.1038/sj.bjc.6690097>.